Environmental Health Perspectives
Vol. 67, pp. 17-24, 1986

Identification of the Mutagens in Cooked Beef

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The purification of cooking mutagens depends on the extraordinary sensitivity of the Ames/Salmonella mutagenicity test and its usefulness for tracking the mutagens during the purification steps. Following aqueous/acid (pH 2) extraction of fried ground beef (cooked at 200, 250, or 300°C), XAD-2 column adsorption and elution with acetone, and acidic and basic liquid/liquid extractions, the samples are separated into six distinct peaks with preparative reverse-phase HPLC. A total of nine distinct mutagens can be separated after two additional HPLC steps. These compounds fall into a class of compounds called aminoimidazoazaarenes (AIAs). The majority of the mutagenic activity is made up of MeIQx[1] (*m/z* 213, $C_{11}H_{11}N_5$), DiMeIQx (*m/z* 227, $C_{12}H_{13}N_5$), trimethylimidazopyridine (TMIP) (*m/z* 176, $C_9H_{12}N_4$) and phenylimidazopyridine (PhIP) (*m/z* 224, $C_{13}H_{12}N_4$). Smaller contributions are from IQ (*m/z* 198, $C_{11}H_{10}N_4$), MeIQ (*m/z* 213, $C_{12}H_{12}N_4$), a nonpolar peak containing oxygen and two unidentified trace polar mutagens. Mass estimates (per kilogram uncooked beef) include: 15 μg for PhIP, 1.0 μg for MeIQx, 0.5 μg for DiMeIQx, and 0.02 μg for IQ. Because of the uncoupling of mutagenic and carcinogenic potencies of these aromatic amines, the PhIP, which contributes the highest mass content to the cooked meat, but has the lowest mutagenic potency, might ultimately make a significant contribution to the carcinogenicity.

Introduction

The production of mutagenic aromatic amines in cooked protein-containing foods was first described by Sugimura et al. (*1*). A year later Commoner and his coworkers deduced that the elevated background of revertants found with the standard Ames/Salmonella mutagenesis assay was due to the beef extract used to grow the bacterial culture (*2*). It was also noted by this group and by Felton and Nebert (*3*) a few years earlier that metabolizing enzymes were needed for this increased background. It was also found that other cooked beef products such as hamburger had mutagenic constituents (*2*). Since these early findings our laboratory has examined various cooked protein food sources for mutagenicity and we have found mutagenic activity in extracts of muscle of beef, lamb, chicken, pork, and fish (*4*). Meat derived from organs such as liver and kidney showed very low mutagenicity, as did nonmeat protein-derived cooked foods such as beans, cheese, and tofu (*5*).

The risk of eating or inhaling biologically active complex mixtures is a combined risk of the potency of the active material and the total mass of the material present. Purification is then necessary for determining the mass of each biologically active component present. This is especially true when the biologically active material ranges in specific activity over more than three orders of magnitude, as has been seen with the cooked food mutagens amino-α-carboline (AaC) (200 rev/μg) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) (660,000 rev/μg) (*6*).

Once the mass and the specific activity of the major components are known, the risk to a specific population, such as the U.S. population eating well-done beef, can begin to be estimated. These calculations are based on the mass of each major mutagen consumed over a lifetime and the extrapolated animal carcinogenicity or cellular mutagenicity of the compounds present.

General Approach to Purification

The question then is how can one best identify and measure the mass of each individual mutagen present

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in a complex matrix at 0.1 to 10 ppb concentrations? We have found that the purification can be monitored by the Ames/Salmonella test quite readily. The identification of a few mutagenic HPLC fractions out of 100 is rather easy, although each assay requires 48 hr.

For compounds with high specific activity (mutations per nanomole) the active material can be measured below the part per billion range. The challenge then is to purify and identify the active component(s) from a matrix of 10^9 molecules of inert material for every molecule of active mutagen. Once purified, there has to be enough mass available (10–100 ng for mass spectral analysis and 10 to 100 μg for NMR analysis). This then necessitates starting with 10 to 100 kg of original material when the active components are present in the parts per billion range and undergoing minimal losses during the purification process.

Identification of Specific Mutagenic Compounds from Ground Beef

A number of mutagens, Trp-P-1, AaC, MeAaC, MeIQx, and IQ (Table 1) have been identified (*7–9*) using various degrees of analytical chemical stringency in cooked beef prepared under a variety of conditions. An extensive effort has been underway in our laboratory to determine the composition of the mutagens in fried ground beef. We have been able to confirm the presence of both MeIQx and IQ, although IQ is present in only trace amounts. The other three compounds, Trp-P-1, AaC, and MeAaC are not detectable in the ground beef cooked at 200 or 300°C. Instead, a number of new compounds have been found using the basic approach described previously (*10*). They include DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline), TMIP (2-aminotrimethylimidazopyridine), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5,*b*]pyridine) (*11*). In addition, trace amounts of MeIQ (2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline) are present at the higher cooking temperatures.

The bulk of this article will deal with the major mutagens isolated from fried ground beef. These mutagens fall into three groups (imidazoquinolines, imidazoquinoxalines and imidazopyridines), all members of a general class of compounds we will call aminoimidazoazaarenes (AIAs) (Fig. 1). These compounds all have an imidazo group with an amino moiety on the 2 position. They also all have a methyl group on one of the ring nitrogens in the imidazo ring and at least one aromatic ring is fused to the imidazo ring for all of the compounds in the AIA class. Their resistance to deamination (due to the guanidyl constituent in the imidazole ring) following nitrite treatment under acidic conditions (*6,10*) sets them apart from the high temperature amino acid pyrolysis products (Trp-P-2, Trp-P-1, AaC, MeAaC, Glu-P-1, and Glu-P-2) (*6*), which are nitrite-sensitive.

Table 1. Abbreviations used.

Abbreviation	Mutagen
AaC	2-Amino-9H-pyrido[2,3-*b*]indole
DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
Glu-P-1	2-Amino-6-methyldipyrido[1,2 – a,3′,2′-d]imidazole
Glu-P-2	2-Aminodipyrido[1,2 – α:3′2′-d]imidazole
IQ	2-Amino-3-methylimidazo[4,5-*f*]quinoline
MeAaC	2-Amino-3-methyl-9H-pyrido[2,3-*b*]indole
MeIQ	2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline
MeIQx	2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5,*b*]pyridine
TMIP	2-Amino-*N*,*N*,*N*-trimethylimidazopyridine
Trp-P-1	3-Amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole
Trp-P-2	3-Amino-1-methyl-5H-pyrido[4,3-*b*]indole

Metabolic Activation and Genotoxic Effects

For IQ (*12*), and more than likely the other AIAs as well, the metabolic activation depends on hydroxylation of the 2-amino group on the imidazole ring. This class of compounds includes one of the most potent mutagenic compounds tested in the Ames/Salmonella assay, MeIQ, (*13,14*) and a compound positive for tumors in mice and rats, IQ (*15,16*). The prevention of DNA damage and carcinogenicity from such compounds can be initially studied by altering the parameters of formation. For example, the amount of aromatic amines isolated from fried meat increases with time and temperature of cooking (*17*). In fact, specific mutagens increase at the higher cooking termperatures (*18*). Knowledge of the specific mutagenic components also allows one to study the effect of natural inhibitors of metabolic activation and/or interaction with the active intermediates. In addition, quantities of the specific mutagenic components isolated from the complex mixture must be identified without ambiguity so that sufficient quantities can be synthesized for use in more comprehensive carcinogenic and mutagenic evaluations.

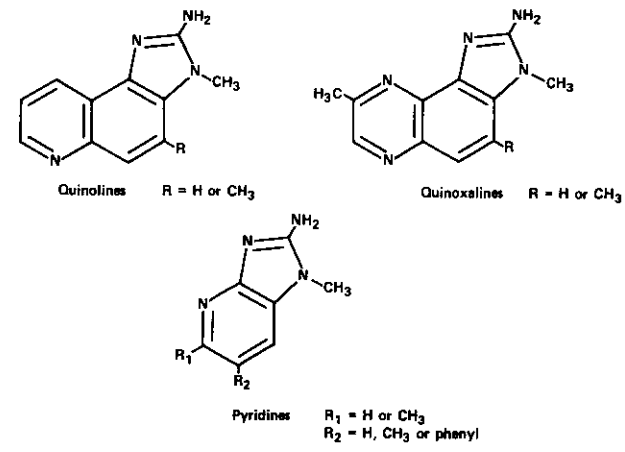


FIGURE 1. Three classes of aminoimidazoazaarenes (AIAs) found in cooked ground beef.

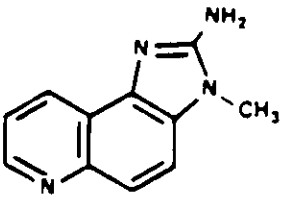
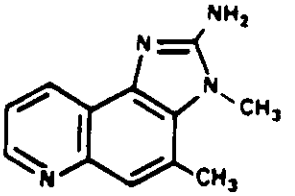
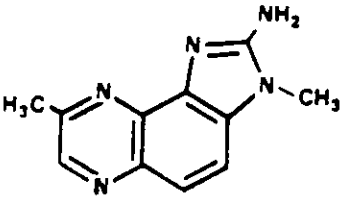
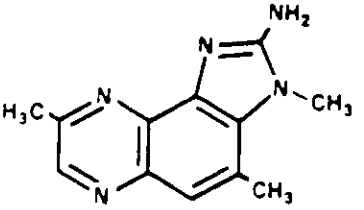
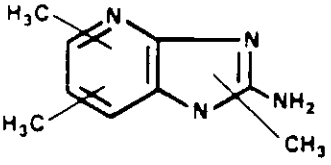
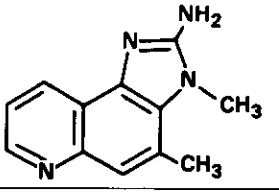
Isolated Beef Mutagens

The major mutagens listed below have been purified from 100 kg batches of fried ground beef formed into patties (1.5 cm × 9 cm) and cooked at either 250°C or 300°C for 6 min per side (see Table 2). Patties were cooked well-done but not charred on a feedback-controlled stainless steel griddle (*19*). Three of these mutagens MeIQx, DiMeIQx, and PhIP, have been purified in sufficient mass for NMR spectral analysis. The current status of each mutagen will be discussed in order of elution from a preparative 10 μm PRP-1 HPLC column (*10*). Figure 2 shows the profile generated by Ames/Salmonella testing (TA 1538) of the fractions collected for 2 min each. The order of reverse-phase elution from the HPLC is from polar to nonpolar compounds.

Peaks I and II (Polar Components)

These mutagens make up a small percentage of the total mutagenic activity (ca. 3%) (Table 3). Peaks I and II resolve into three primary peaks upon further HPLC chromatography. The major one (peak II) has been purified close to homogeneity and has a molecular weight of 202. A mass fragment with a weight of 187 indicates loss of a methyl group which is typical for the entire class of food mutagens. The high resolution mass spectrum did not give a clear composition fit for the molec-

Table 2. AIA mutagens isolated from fried ground beef.

Compound	MW	Concn, μg/kg	Structure
IQ 2-Amino-3-methylimidazo[4,5-*f*]quinoline $C_{11}H_{10}N_4$	198	< 0.1	
MeIQ 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline $C_{12}H_{12}N_4$	212	Trace	
MeIQx 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline $C_{11}H_{11}N_5$	213	1.0	
DiMeIQx 2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline $C_{12}H_{13}N_5$	227	0.5	
TMIP 2-Amino-*N*,*N*,*N*-trimethylimidazopyridine $C_9H_{12}N_4$	176	0.5	
PhIP 2-Amino-1-methyl-6-phenylimidazo[4,5,*b*]pyridine $C_{13}H_{12}N_4$	224	15	

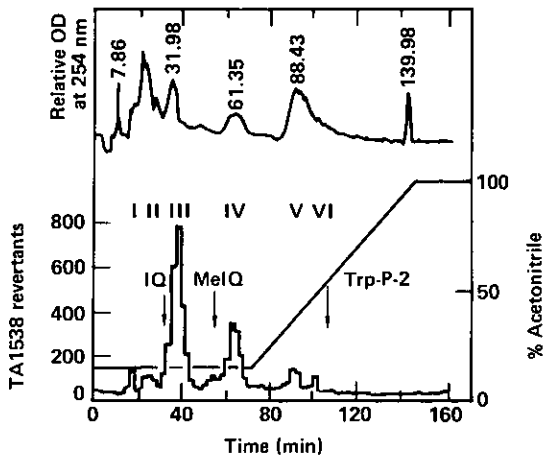


FIGURE 2. Preparative reverse-phase HPLC separation. The PRP-1 column was eluted with a flow rate of 2.5 mL/min using a mobile phase of (*A*) water and (*B*) acetonitrile with 0.1% diethylamine in both solvents. Initial separation was isocratic at 15% B for 70 min, followed by a 1.2% per min gradient for an additional 70 min; 2 min fractions were collected throughout the run. Approximately 400 mg of sample dissolved in 15% acetonitrile was injected in a volume of 5.0 mL. An aliquot of 0.2% of each fraction was analyzed with the Ames/Salmonella test. The left ordinate scale is TA 1538 revertants/plate. Reprinted with permission (*10*).

ular ion and its fragments, so the elemental composition is not yet known. Peak II has an approximate Ames/Salmonella activity of 30,000 revertants per microgram (TA 1538). Further work is in progress to characterize this molecule. The other polar mutagens (peaks Ia and Ib) have not yet been purified to single components.

Peak IIIa (MW = 213), MeIQx

This mutagen (MeIQx) is a major source of mutagenic activity in fried ground beef (see Table 3). It contributes more than 20% of the total mutagenic activity of the 300°C cooked beef. MeIQx has a specific activity in the Ames/Salmonella assay (TA 1538) of 70,000 rev/μg. Approximately 1.0 μg/kg equivalent of uncooked ground beef is present. MeIQx has a UV absorption maximum at 274 nm. The mass spectrum matched both that reported in the literature (*20,21*) and that for the synthetic MeIQx (a kind gift from Dr. Nishimura) analyzed in our laboratory. The parent ion is *m/z* 213. Major fragments corresponding to losses of H, CH_3, H + HCN and HCN + CH_3CN could be observed at *m/z* 212, 198, 185, and 145, respectively.

Enough material (> 20 μg) was purified to homogeneity and examined for an NMR spectrum. The spectrum from the purified material agrees with the spectral analysis both at LLNL and in Japan of the synthetic material prepared by Dr. Nishimura and his co-workers (see Table 4). From all of the criteria examined it seems clear that the same isomeric form of MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline) is present in our 300°C cooked ground beef (peak IIIa fraction) as is present in the synthetic MeIQx.

Peak IIIb (MW = 176), TMIP

Peak IIIb, a second peak separated by normal-phase HPLC from peak III, contributes approximately 15% of the total mutagenic activity from the fried beef (see Table 3). This material has a UV absorption spectral maximum at 299 nm. The specific mutagenic activity is 100,000 rev/μg and approximately 0.5 μg of TMIP is produced after frying 1 kg (fresh weight) of ground beef. Peak IIIb has a molecular weight of 176 and mass fragment ions at *m/z* 175, 161, 160, 148, and 134 corresponding to losses of H, CH_3, NH_2, CH_2N, and CH_2N_2, respectively. At the present time, not enough material has been recovered in pure form for NMR spectral analysis, but there are enough preliminary data to suggest a trimethylimidazopyridine structure for peak IIIb. This includes work in our laboratory with 2-aminotrimethylbenzimidazoles that has shown these two-ring molecules to be quite mutagenic. The synthesis of one of the 12 possible isomers of the TMIP series (2-amino-*N*-methyl-4,6-dimethylimidazopyridine) has demonstrated that these compounds can be mutagenic. This molecule has an identical mass spectrum to peak IIIb; however, it has a much lower specific activity and does not coelute on HPLC. This work supports our proposed general structure for peak IIIb. Based on structure/activity predictions we are proceeding with additional syntheses of specific members of the TMIP series

Table 3. Mass and mutagenic contribution of individual beef mutagens fried at 300°C.

Compound	MW	Specific activity, 10^3 rev/μg	Mutational activity, rev/kg,	% Mutational activity
IQ	198	200	≈20,000	~6.0
MeIQ	212	700	≈14,000	~4.2
MeIQx	213	70	70,000	21.1
DiMeIQx	227	130	65,000	19.7
TMIP	176	100	50,000	15.0
PhIP	224	4	60,000	18.1
Peak V	216	—	43,000	1 3.0
Polar Peaks	—	—	10,000	3.0
Total			332,000	100.0

Table 4. MeIQx NMR spectrum.

Position	Chemical shift, ppm		
	Peak IIIa[a]	MeIQx[a]	MeIQx[b]
7-H	8.68	8.68	8.65
5-H	7.76	7.75	7.75
4-H	7.58	7.59	7.51
NH_2	6.60	6.58	4.88
3-Me	3.69	3.69	3.69
8-Me	2.72	2.72	2.80

[a] Proton shifts in DMSO-D6 (LLNL).
[b] Proton shifts in $CDCl_3$ (Japan).

with the hope that the exact structure of the peak IIIb congener can be elucidated.

Peak IIIc (MW = 198), IQ

Peak IIIc, a third peak separated by normal phase HPLC of peak III contributes only 6% of the total mutagenicity (Table 3). IQ is a potent mutagen with more than 200,000 rev/μg formed in strain TA 1538. The compound purified from the fried beef has a mass spectrum identical to synthetic IQ (*10*) and coelutes on both reverse-phase and normal-phase HPLC with ^{3}H-labeled IQ. The amounts present in 300°C cooked meat are far too small (1000 kg of meat would be needed with a 20% yield) for NMR determinations. Our laboratory has not found any preferential increase in IQ concentration with higher fat content of the meat (*18*).

Peak IVa (MW = 212), MeIQ

This peak appears as a leading shoulder or a small peak in front of the main peak IV. It becomes quite pronounced at higher cooking temperatures (see Fig. 3, 300°C thin) and is clearly separated from peaks III and IV. Synthetic MeIQ coelutes with peak IVa following reverse- and normal-phase HPLC. With the latest purification, we have been able to confirm this identification with mass spectral analysis (see Fig. 4). Since MeIQ is such a potent Ames/Salmonella mutagen, a very large number of revertants is needed for enough mass to be present for either mass spectral determinations or NMR analysis. Since 100 kg of meat generates upon cooking only 1 or 2 μg of MeIQ and since final yield cannot be expected to be much better than 20%, then NMR analysis (20 μg minimum purified material) would take 10,000 kg of beef needing a commercial scale (pilot plant) purification. This seems an unreasonable effort and is not likely to be carried out.

Peak IVb (MW = 227), DiMeIQx

This new mutagen was first reported by our laboratory to be a DiMeIQx congener (*10*). It is the major

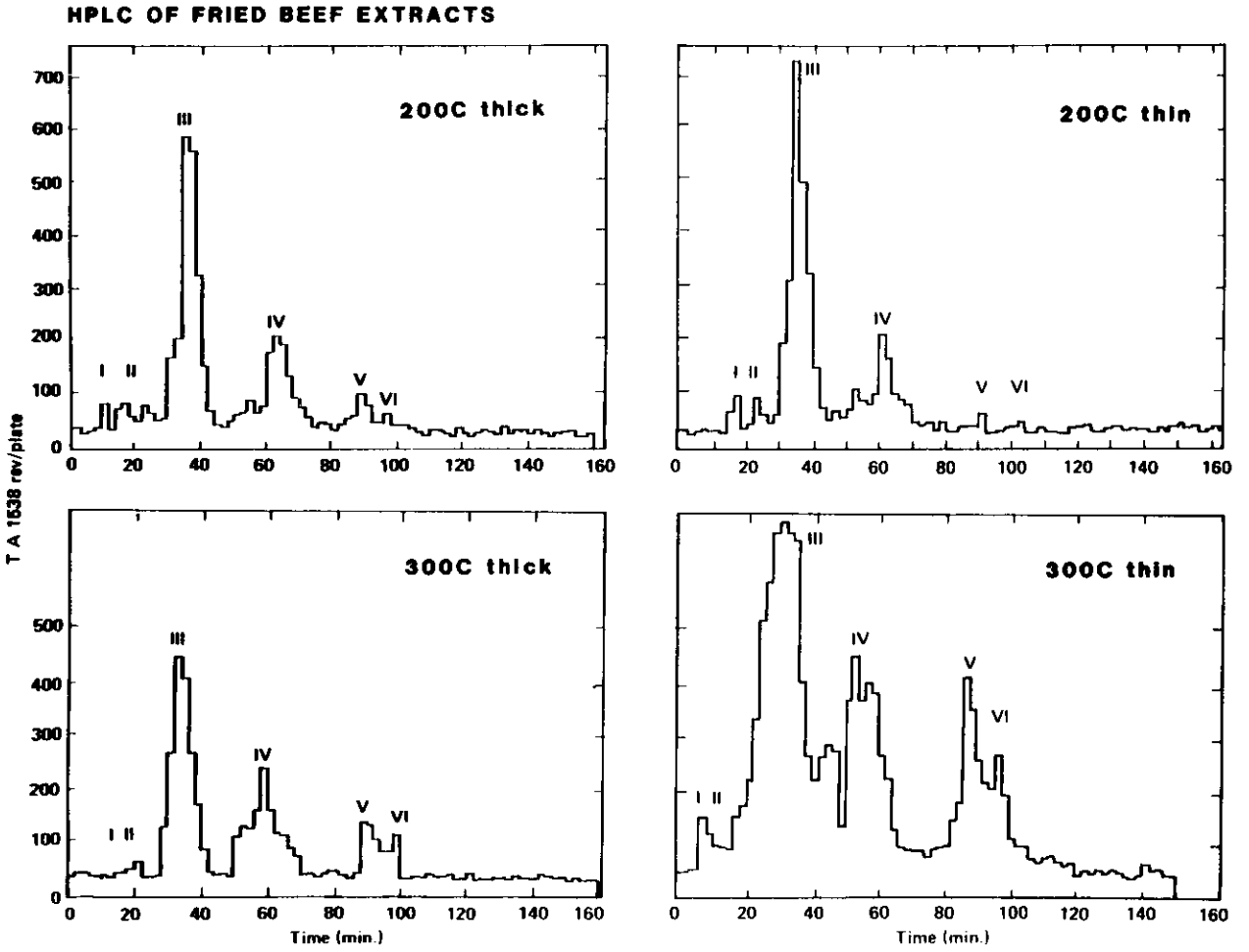


FIGURE 3. Ames/Salmonella activity profiles of the HPLC separation (see Fig. 2 for conditions) of beef extracts. (*a*) thick patties fried at 200°C, 0.20 kg-eq tested; (*b*) thin patties fried at 200°C, 0.003 kg-eq tested; (*c*) thick patties fried at 300°C, 0.003 kg-eq tested; (*d*) thin patties fried at 300°C, 0.005 kg-eq tested. All fractions were tested with S9 at 2 mg/plate, background (15–35 rev/plate) not subtracted. (Reprinted with permission) (*18*).

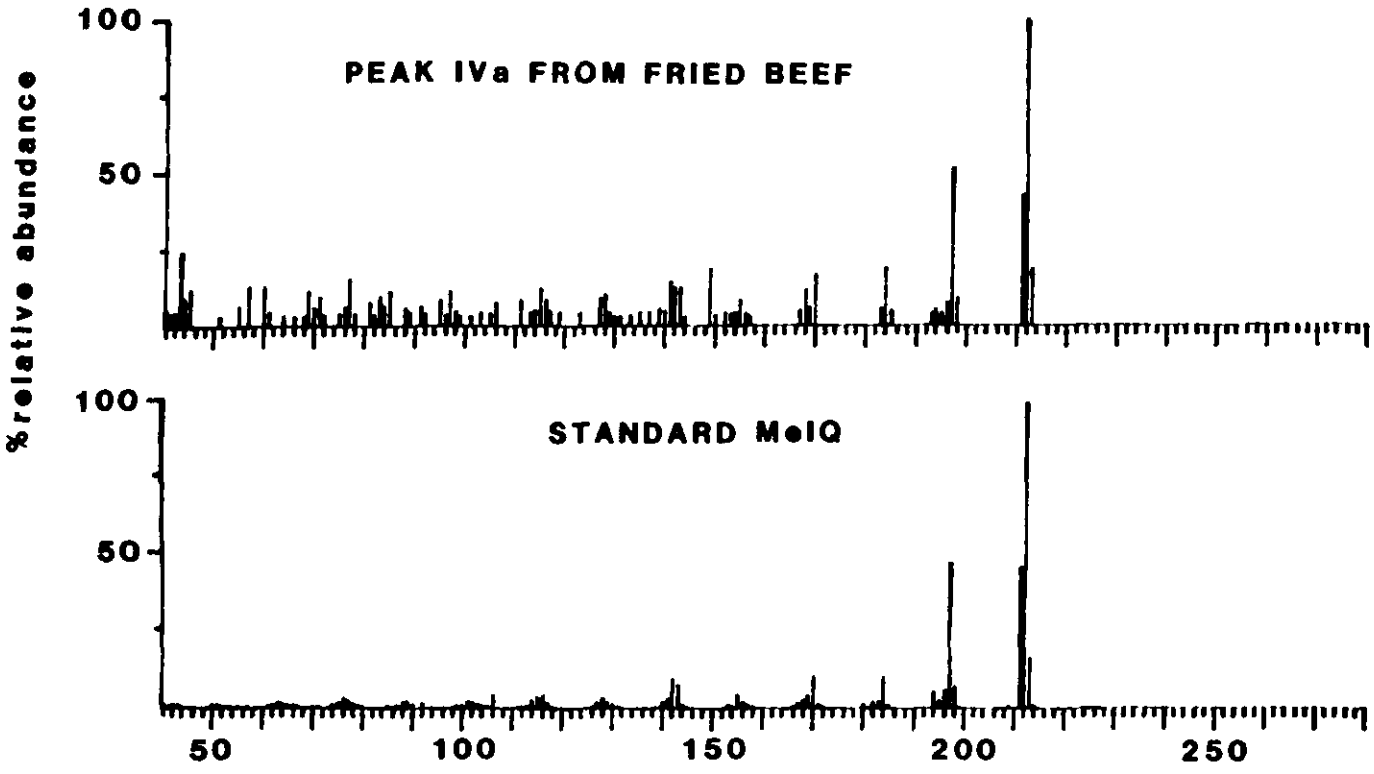


FIGURE 4. Low resolution mass spectra: (top) Peak IVa; (bottom) synthetic MeIQ.

mutagenic component in peak IV and contributes approximately 20% of the total mutagenicity to the cooked beef. It is present at about 0.5 μg/kg wet weight equivalent of beef and has a specific activity almost double that of MeIQx, or 130,000 rev/μg (TA 1538). Peak IVb upon purification gave a UV maximum absorption at 274 nm. Mass spectral analysis gave a parent ion at 227 and fragment ions with *m/z* values of 226, 212, and 199 which correspond to losses of H, CH_3, and/or NH, and CH_2N, respectively.

Enough sample was recovered from repeated 100 kg fried beef purifications that we were able to get definitive NMR spectra with three different solvents. The spectra are definitely consistent with that published for synthesized 4,8-DiMeIQx (*22*) (see Table 5). They are also consistent with the C-methyl groups being on opposite sides of the molecule (i.e., either 4 or 5 and either 7 or 8 positions). The NMR data are theoretically not inconsistent with the 4,7; 5,7; or 5,8 methyl isomers, either. It thus appears that the exact structural elucidation of the purified material (peak IVb) will have to await synthesis of all four possible isomers.

Peak V (MW = 216)

This nonpolar peak has not been purified to homogeneity but does have the majority of its mutagenic material associated with a 216 molecular weight compound. High-resolution mass spectrometry suggests the presence of an oxygen atom in this molecule. The oxygen appears to be incorporated into a ring because of the nonpolar nature of the compound as well as the lack of oxygen containing fragments following ionization in the mass spectrometer. The mutagenic activity of this peak contributes approximately 13% of the total Ames/Salmonella activity of fried beef.

Peak VI (MW = 224), PhIP

This peak is the most nonpolar of the mutagens isolated from cooked ground beef. It has a molecular weight of 224 and a specific Ames/Salmonella activity of only 4,000 rev/μg (TA 1538), the weakest of the isolated beef mutagens. The interesting thing about this mutagen is that it is present in such high mass compared to the other AIAs (see Table 2). Approximately 15 μg/kg wet weight equivalent is present after cooking at 300°C. This is 18% of the total mutagenicity and most of the mass (8 times the total mass of the other mutagenic components). This mutagen, peak VI, and peak

Table 5. Comparison of Peak IVb and DiMeIQx NMR proton shifts in $CDCl_3$.

Position	Peak IVb	3,4,8-DiMeIQx[a]
7-H	8.62	8.63
5-H	7.46	7.46
3-Me	3.91	3.92
4-Me	2.84	2.83
8-Me	2.79	2.79

[a] Data of Grivas (*22*).

V as well, increase more in proportion to the other mutagens at the higher 300°C cooking temperatures (Fig. 3) (*18*). Peak VI has a very distinct UV absorption maximum at 316 nm. The shift to the higher wavelength is most likely due to the phenyl group.

Mass spectral analysis reveals little about the structure with fragments at *m/z* 223, 217, 208, 196, and 170. In addition a fragment was seen at *m/z* 77, possibly due to the phenyl group. Because of the high concentration of this material in the meat an ample amount (50 μg) has been purified for determining NMR spectra.

The spectrum was consistent with a 2-amino-(*N*-1 or *N*-3)-methyl-6-phenylimidazo[4,5,*b*]pyridine [proton NMR (d_6 acetone): δ 3.70 (N-Me,s,3H), 6.32 (NH_2, broad S, 2H), 7.31 (4′-H,m,1H), 7.45 (3′,5′-H,m,2H), 7.67 (2′,6′-H,m,2H), 7.69 (4-H,m,1H), 8.36 (6-H,d,1H)]. In fact, 2-amino-1-methyl-6-phenylimidazo-[4,5,*b*]pyridine has been synthesized in our laboratory and appears identical to Peak VI based on a number of criteria. The Ames/Salmonella specific activity, UV absorption maximum, nitrite sensitivity, and mass spectrum are identical. The NMR spectrum also suggests identity. Synthesis of the 3-methyl isomer is nearly complete in our laboratory and preliminary comparisons suggest the 3-methyl isomer is not present in peak VI. In addition, we are examining the nuclear-overhauser effect (NOE), in this case, the interaction of the *N*-methyl protons with a neighboring ring proton. The position of the *N*-methyl group can then be located and should establish the exact structural isomer present in Peak VI.

Discussion

The ultimate fate of the AIAs upon ingestion will depend on their metabolism, transport and stability in the body. Felton and Healy (*23*) examined the metabolism of an extract containing all of the AIAs for its ability to be converted to Ames/Salmonella positive mutagens by human liver microsomes. Microsomes from all individuals in the study (six) showed appreciable conversion of the AIA extract to mutagenic intermediates. In one case, the mutagenic activity was nearly as high as that activated by Aroclor-induced rat microsomes. The study indicates for a very small human sample that the human is normally induced for the proper monooxygenases (cytochrome P-450s) to convert the AIAs to active bacterial mutagens. Through studies where human fluids (*24,25*) and feces (*26*) were examined for mutagens after consumption of cooked meats we can conclude that the mutagens and/or their metabolites are stable enough as they pass through the intestinal tract, liver, blood, and kidneys to be detected upon excretion.

It seems clear that the total amount (mass) of these fried beef mutagens (AIAs) in a kg equivalent of beef is quite small. The work described in this report, primarily from our laboratory, indicates that approximately 20 μg of mutagen is present in a kilogram of beef fried at 300°C. Genetic toxicology data from cells in culture (*27,28*) and mice (*29*) suggests that one of the AIAs, IQ, is not as potent in these systems as one might expect from its potency in the Ames/Salmonella or other microbial mutagenesis assays (*30*). Loury and Byard (*31*), though, have seen a good response of unscheduled DNA synthesis with IQ in hepatocytes, as have Terada and his co-workers (*32*) with mutagenesis in Chinese hamster lung cells. In addition, Ohgaki and her co-workers (*15*) and Takayama and his co-workers (*16*) have seen tumors at several sites in mice and rats with IQ. Thus, at least for IQ, predictions of genotoxic and carcinogenic potency based on Ames/Salmonella response are very difficult. It is also hard to predict how the behavior of IQ will mimic that for the other AIAs in cells, in whole animals, or least of all in man. But it is important to note that IQ is the first of the AIAs to be studied in animal tumor bioassays and shows a positive response similar to the carbolines and azacarbolines in long-term feeding studies in both mice and rats (*6,8,33,34*).

The difficulty of making human risk assessments must not be underestimated, but very preliminary risk estimates can be made by assuming consumption of a certain amount of cooked meat per day and then calculating the dose of AIAs ingested. This human lifetime dose then can be compared to the animal TD_{50} (the dose to give half the animals cancer in a lifetime) for IQ, for example. Suppose that a 60 kg human ate 250 g of well-done meat a day (80 ng/kg/day AIA based on 20 μg AIA/kg ground beef) for a lifetime of 70 years = (2 mg/kg/lifetime). Based on an estimated TD_{50} for a mouse of 15 mg/kg/day (*35*) or 10 g/kg/lifetime estimating a 2-year life span, the difference between the dose required for the mouse TD_{50} and the human dose is 5000-fold. This difference of 5000-fold between man and mouse is only a gross estimate and includes a number of assumptions; but it does suggest that a significant fraction of human cancer could be the result of consuming AIAs from cooked meat.

The authors thank Susan Healy and Anita Avery for their technical expertise with the Ames/Salmonella test. The authors would also like to thank Karl Grose for cooking the beef and Chuck Morris for performing the low resolution mass spectrometry. Special thanks are due Leilani Corell and Verna Steckel for preparation of the manuscript.

Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-ENG-48 and the Department of Nutritional Sciences, University of California, and supported by an Interagency Agreement (NIEHS 222Y01-ES-10063) between the National Institute of Environmental Health Sciences and the Department of Energy.

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